Advanced Science Letters
Vol. 23, 3372–3375, 2017

Annona muricata Associated with Reduced Macrophage Phagocytic Index of Swiss Mice During Cerebral Malaria Phase

Kis Djamiatun[1,2,*], Husen Mohamed Albakoush[2], Tri Nur Kristina[3], and Djoko Nugroho[4]

[1] *Parasitology Department Medical Faculty Diponegoro University, Semarang, 50275, Indonesia*
[2] *Biomedical Science Post Graduate Program of Medical Faculty Diponegoro University, Semarang, 50231, Indonesia*
[3] *Medical Faculty Diponegoro University, Semarang, 50275, Indonesia*
[4] *Biostatistic Department Public Health Faculty Diponegoro University, Semarang, 50275, Indonesia*

Background: Cerebral malaria (CM) is mostly fatal case. Anti-plasmodial and immunomodulatory effects of *Annona muricata*-leaf extract (AME) might avoid CM. Immunomodulatory treatment which increased macrophage-phagocytic index (PI) and macrophage nitric oxide (NO) production, related to the increase survival of experimental (E)CM. The objectives were to determine whether ethanolic-AME influenced PI, NO-production, and correlate PI and NO-production with parasitemia during CM-phase. *Method*: A post-test only control group design-study was done. Thirty swiss-mice were randomly divided into 6 groups, PbA-inoculated and healthy mice grouped in C(+) and C(−); healthy mice treated with *A. muricata* 100 and 150 mg/Kg BW/day named as X_1 and X_2; PbA-inoculated and treated either dose mentioned above grouped as X_3 and X_4. Light microscope was used to observe parasitemia and PI. NO was measured using elisa. One-Way Anova and Benfereoni-post-hoc test were performed for normally-distributed data. Pearson test was done for analyzing correlation between variables. *Results*: Parasitemia-percentage and NO production were not different among PbA-inoculated groups ($p = 0.916$ and $p = 1.000$). NO produced in each of C(+), X_3 and X_4 group was significantly lower than C(−) ($p < 0.0001$). PI of X_3 was significantly lower than C(−) group ($p = 0.022$). Activated-macrophage NO production correlated strongly with parasitemia-percentage in X_3 group ($r = 0.852$, $p = 0.015$). *Conclusion*: The conclusions were AME treatment at any dose studied might not improve NO-production and decrease parasitemia-percentage of swiss mice during CM phase. Dose of 100 mg/kg BW/day AME might reduce PI below normal during CM-phase. This dose might contribute to a strong correlation between NO production and parasitemia percentage.

Keywords: *Annona muricata*, Cerebral Malaria, Macrophage, NO.

1. INTRODUCTION

Malaria caused 584,000 deaths out of 198 million malaria cases in 2013. Fatal malaria cases associated severe malaria (SM) including cerebral malaria (CM) and severe malaria anemia (SMA).[1]

The SM pathogenesis is associated with many factors including high parasitemia, immunopathology, vascular endothelial cell dysfunction and multi-organ dysfunction.[2] Parasitemia and immune responses against *Plasmodium* such as *P. berghei* ANKA (PbA) are essential for the development of experimental cerebral malaria (ECM).[3] Immune responses which control parasitemia, also contribute to the development of ECM.[4–6] Interestingly, immunomodulator treatments prevent PbA-inoculated mice from ECM.[4,7] The development of ECM was prevented due to reduce both the sequestration of iRBC and the altered of CD8+ T cells activation observed in the brain. Immunomodulator which stimulates the increase of macrophage phagocytic index and mean of nitric oxide (NO) production by macrophages is associated with longer survival time.[8] Additionally, an immunomodulator which increases phagocytic activity which contributed to the clearance of parasitized red blood cells (pRBCs) but decreases inflammatory respond to *Plasmodium sp*-infection and enhances the survival rate of ECM mice model.[9] Phagocyte-derived reactive nitrogen species are not essential for PbA elimination in mice models.[10] Exogenous NO donor, however, prevented ECM since the treatment eased edema, leukocyte accumulation and hemorrhage incidence in the brain. S-nitrosoglutathione (GSNO), a physiologically NO donor, is able to prevent ECM development in a wide range of doses, decreasing brain inflammation and inducing milder cardiovascular side effects.[11] Traditional medicine, mainly traditional plants, is used as primary health

*Author to whom correspondence should be addressed.

1936-6612/2017/23/3372/004 doi:10.1166/asl.2017.9171

care in substantial number of people in developing country. Traditional herbs for malaria treatments were found in several countries. Numbers of potential traditional herbal medicine used as effective modern medicine in several diseases including malaria.[12] *Annona muricata* is commonly used for the treatment of malaria. *In vitro* study showed the susceptibility of *Plasmodium falciparum* (*P. falciparum*) to *Annona muricata* extracts, which was mediated by the effect of acetoginin and other active compounds which interfered important enzyme of *Plasmodium*.[13] Anti malaria of water based *Annona muricata* leaf extract (AME) was noticeable in *P. berghei* ANKA (PbA)-inoculated mice.[14] Ethanolic-AME treatment to PbA-inoculated Swiss mice, experimental (E)CM susceptible mice, associated with reduces tumor necrosis alpha (TNF-α) and a pro-inflammatory cytokine, and increase inducible splenic-NO production during CM phase.[15] Protective effect of ethanolic-AME was further elucidated in recent study by analyzing macrophage phagocytic index, NO production, and parasitemia of CM phase Swiss mice treated with *Annona muricata.*

2. METHOD

In the post test only control group design was performed in this study by using 36 Swiss mice. Ethical clearance was provided by Ethic committee of Medical Faculty, Diponegoro University-Dr. Kariadi Hospital (No. 426/EC/FK-RSDK/2015). The mice were divided into 6 groups that were negative control coded as C(−) and included healthy mice without any treatment. X_1 and X_2 groups were healthy mice treated with AME dose of 100 and 150 mg/kg BW, respectively. Positive control coded as C(+) group which inoculated with PbA in dose of 10^7 pRBCs. X_3 and X_4 groups were treated with AME in ether dose and inoculated with PbA. Phagocytic index = (percentage of macrophages containing at least one latex bead) × (mean number of bacteria per positive cell). NO level measured on the supernatant of macrophages exposed to the latex bead. Total NO level then was measured by the Elisa method, using Griess reagent I and II. Parasitemia was counted from ratio between pRBCs and total erythrocyte. Statistical analysis was done by using Oneway ANOVA for normally distributed data which was homogen. Post hoc analysis was used after One-way ANOVA have statistical differences. Correlation test between two variables used Pearson Test.

3. RESULTS

3.1. Phagocytic Index of Peritoneal Macrophages

Bonferroni as post hoc test following One-Way ANOVA showed that PI of C(−) group was not different than those of C(+) and X_4 groups ($p = 0.136$ and $p = 1.000$, respectively). Interestingly, PI of C(−) group was significantly higher than those of X_3 group ($p = 0.022$). PI of C(−) group was significantly higher than those of X_1 and X_2 ($p = 0.026$ and $p = 0.001$, respectively). PI of X_1 group was not different with X_2 group ($p = 1.000$). PI of X_1 group was not different than X_3 group ($p = 1.000$). No difference in phagocytic index between X_2 and X_4 groups was also observed ($p = 0.073$). This suggested that two AME doses had similar effect toward phagocytic index of healthy and CM-phase swiss mice.

3.2. NO Produced by Peritoneal Macrophages

NO levels in each studied group were normaly distributed (Shapiro-Wilk test, $p > 0.05$). Test of homogeneity of variance showed that each studied group was homogene (Levene test, $p > 0.05$). One way anova showed significant difference ($p < 0.0001$), then Benfereoni post hoc test was performed. NO levels of C(−) group were significantly higher than those of either C(+), X_3 or X_4 groups ($p < 0.0001$). NO levels of C(+) groups did not significantly difference than those of either X_3 or X_4 group ($p = 1.000$). Additionally, NO levels of X_3 and X_4 groups were comparable ($p = 1.000$). NO levels of X_3 group were significantly lower than X_1 group ($p < 0.0001$). This was also observed by comparing NO levels of X_4 and X_2 group ($p < 0.0001$). NO levels of C- group were not different with those of X_1 and X_2 groups ($p = 1.000$). Furthermore, NO levels of X_1 and X_2 groups were not different ($p = 1.000$). This suggested that AME treatment in healthy mice were not associated with NO level.

3.3. Parasitemia Percentage of PbA-Inoculated Groups During CM Phase

Parasitemia percentage of C(+), X_3 an X_4 groups was normally distributed in every group based on Shapiro-Wilk test ($p = 0.954$, $p = 0.470$, and $p = 0.929$). Homogeneity of variance test showed homogeneity of parasitemia percentage among C(+), X_3 an X_4 groups ($p = 0.866$). One-way Anova showed that parasitemia percentage was not different among C(+), X_3 and X_4 groups during CM phase ($p = 0.916$).

3.4. Correlation Between PI, NO Levels and Parasitemia Percentage

No correlation between PI, NO levels and parasitemia percentage found in either C(+) or X_4 group. The only correlation shown by Pearson test was that a strong correlation between NO produced by activated macrophage and parasitemia percentage observed in X_3 group ($r = 0.852$, $p = 0.015$).

Figure 1 were box plot of Swiss mice in ethanolic-AME study. Figures A and B were box plots of PI and NO produced by activated peritoneal macrophages, respectively. Figure C. was graph of parasitemia level. C(−), X_1 and X_2 groups were healthy Swiss mice. C(−) group received no AME treatment, while X_1 and X_2 groups were treated with AME 100 and 150 mg/kg BW/day, respectively. C(+), X_3 and X_4 groups were PbA-inoculated Swiss mice. C(+) group received no AME treatment without AME treatment. X_3 and X_4 groups treated with AME 100 and 150 mg/kg BW/day, respectively.

4. DISCUSSION

Dose of 100 mg/kg BW/day AME associated with reduced PI below normal during CM phase. Phagocytic activity indicated that AME treatment associated with inhibition of phagocytic activity in healthy swiss mice. AME dose suggested that the two AME doses studied had similar effect toward phagocytic index of healthy and CM-phase swiss mice. Immunomodulatory treated PbA inoculated mice showed longer survival time, and associated with higher mean of macrophage phagocytic index, and mean of nitric oxide production by macrophages than those of the non-treated PbA infected mice.[7] Together with recent finding, it may

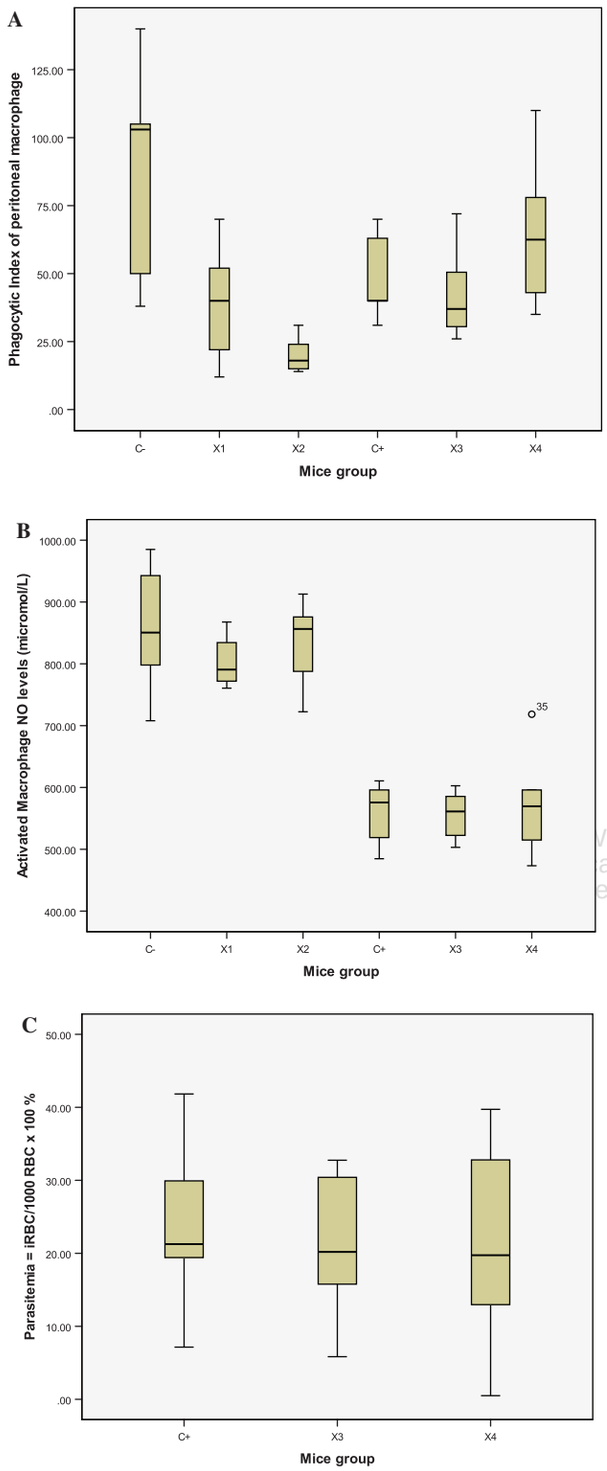

Fig. 1.

be that AME protective effect is not mediated by increasing PI in CM phase Swiss mice.

By comparing negative control and each group inoculated with PbA (positive control, X_3 and X_4 groups), it was found that lower NO levels than normal value was associated CM phase in each PbA-inoculated groups. This indicated that AME at any dose studied might not be able to normalize NO produced by activated peritoneal macrophages of CM-phase Swiss mice. These findings were confirmed by comparing NO produced by X_1 and X_3 showing that a significantly lower NO production of X_3 group ($p < 0.0001$). Similar finding was also found by comparing NO produced by X_2 and X_4 ($p < 0.0001$). Further analyzes between PbA-infected groups, it revealed that AME treatment at any dose studied was not associated with NO level in CM phase. This results was in line with previous studies which observed LPS-induced splenic NO production.[15]

This recent study also showed that AME at any studied dose was not associated with parasitemia level during CM phase. The correlation analyzes was only found in X_3 group. This recent results were not in line with previous *in vitro* studies.[16, 17] It may also be that acetogenin concentration in the extract used in our recent study was not sufficient for controlling PbA infection. There were the different findings in this study than the previous *in vivo* study.[14] This might be due to the difference of mice strain and AME used. The strong positive correlation between NO production and parasitemia ($r = 0.852$, $p = 0.015$) was found in PbA-inoculated mice group which receive 100 mg/kg BW/day at period 7 day before and 7 day post PbA inoculation. This suggested that protective AME contribution during CM phase could not be excluded. However, other protective biomarkers need to be further evaluated during CM phase of AME treated Swiss mice.

5. CONCLUSION

The findings related to peritoneal macrophages and parasite load during CM phase of Swiss mice, conclude that AME might contribute to inhibit PI. Secondly, AME might not be able to increase NO produced. Finally, AME at any dose studied might not able able to control PbA infection

Acknowledgments: Medical Faculty Diponegoro University for finance the publication of this recent study. AME was obtained for free from SidoMuncul Company who also analyzed AME contain.

References and Notes

1. W. H. O. Severe Malaria, Tropical Medicine and International Health: TM and IH, 19(Suppl 1), 7 **(2014)**.
2. S. J. Higgins, K. C. Kain, and W. C. Liles, *Expert Review of Anti-Infective Therapy* 9, 803 **(2011)**.
3. M. L. Wang, Y. H. Feng, W. Pang, Z. M. Qi, Y. Zhang, Y. J. Guo, E. J. Luo, and Y. M. Cao, *Malaria Journal* 13, 116 **(2014)**.
4. A. M. Vigario, E. Belnoue, A. C. Gruner, M. Mauduit, M. Kayibanda, J. C. Deschemin, M. Marussig, G. Snounou, D. Mazier, I. Gresser, and L. Renia, *J. Immunol.* 178, 6416 **(2007)**.
5. J. Palomo, M. Fauconnier, L. Coquard, M. Gilles, S. Meme, F. Szeremeta, L. Fick, J. F. Franetich, M. Jacobs, D. Togbe, J. C. Beloeil, D. Mazier, B. Ryffel, and V. F. Quesniaux, *European Journal of Immunology* 43, 2683 **(2013)**.
6. D. V. Acroute, S. Lafitte, D. Dive, J. Khalife, and C. Pierrot, A role for CD4+ and CD8+ cells and not for CD25+ cells in the control of Plasmodium berghei Anka blood stage parasites in rats, Parasite (Paris, France) 17, 53 **(2010)**.
7. M. I. Muniz-Junqueira, F. O. Silva, M. R. de Paula-Junior, and C. E. Tosta, *Acta Tropica* 94, 128 **(2005)**.
8. L. Serghides, S. N. Patel, K. Ayi, Z. Lu, D. C. Gowda, W. C. Liles, and K. C. Kain, *The Journal of Infectious Diseases* 199, 1536 **(2009)**.
9. S. M. Potter, A. J. Mitchell, W. B. Cowden, L. A. Sanni, M. Dinauer, J. B. de Haan, and N. H. Hunt, *Infection and Immunity* 73, 4941 **(2005)**.
10. G. M. Zanini, P. Cabrales, W. Barkho, J. A. Frangos, and L. J. Carvalho, *Journal of Neuroinflammation* 8, 66 **(2011)**.
11. G. M. Zanini, Y. C. Martins, P. Cabrales, J. A. Frangos, and L. J. Carvalho, *J. Neuroimmune Pharmacol.* 7, 477 **(2012)**.

12. K. Na-Bangchang and J. Karbwang, *Tropical Medicine and Health* 42(2 Suppl), 3 **(2014)**.
13. F. F. Boyom, P. V. Tsouh Fokoua, L. R. Tchokouaha Yamthea, A. N. Mfopaa, E. M. Eugénie Madiesse Kemgnea, W. Fon Mbachamb, E. Tsamoc, P. H. Paul Henri Amvam Zolloa, J. Gutd, and P. J. Rosenthal, *Journal of Ethnopharmacology* 134, 7 **(2011)**.
14. V. Somsak, N. Polwiang, and S. Chachiyo, *Journal of Pathogens* 3264070 **(2016)**.
15. M. E. Karolina, H. Fransisca Prameshinta, E. Dharmana, and K. Djamiatun, *Jurnal Kedokteran Brawijaya* 29, 4 **(2016)**.
16. J. Nguyen-Pouplin, H. Tran, H. Tran, T. A. Phan, C. Dolecek, J. Farrar, T. H. Tran, P. Caron, B. Bodo, and P. Grellier, *J. Ethnopharmacol.* 109, 417 **(2007)**.
17. M. R. Mohd Abd Razak, A. Afzan, R. Ali, N. F. Amir Jalaluddin, M. I. Wasiman, S. H. Shiekh Zahari, N. R. Abdullah, and Z. Ismail, *BMC Complementary and Alternative Medicine* 14, 492 **(2014)**.

Received: 29 September 2016. Revised/Accepted: 23 December 2016.